

**EXPRESS MAIL #EL822582809US**

*Application No.: PCT/US00/19336*

REMARKS

The above amendments are being submitted in connection with the national stage filing of the present Application. Applicants note that the new amendments are based on the status of the claims after entry of amendments made under Article 34 during the PCT phase of the application. The claims have been amended to eliminate the multiple dependent claims from the Application and make other minor amendments as necessary to provide proper antecedent basis for the claims. The amendments to the specification correct some omissions and clerical errors with regard to reference to a sequence identifier.

Applicants also submit herewith a paper copy and computer readable form of the Sequence Listing for the application, pursuant to 37 CFR § 1.821-1.825. All of the sequences in the Sequence Listing were present in the application as filed and the submission of the Sequence Listing adds no new matter to the application. Agent for applicants hereby asserts pursuant to 37 CFR § 1.821(f) that the content of the paper and computer readable copies of SEQ ID NO:1 through SEQ ID NO:87 submitted herewith are identical.

Respectfully submitted,

SHERIDAN ROSS P.C.

By: Angela Dallas

Angela K. Dallas

Registration No. 42,460

1560 Broadway, Suite 1200

Denver, Colorado 80202-5141

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(303) 863-9700

Date: January 14, 2002

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**VERSION WITH MARKINGS TO SHOW CHANGES MADE**

**In the Specification:**

The paragraph on page 22, lines 14-21, has been amended as follows:

--GF-IgG fusion proteins in which the 7 amino acid linker [ser-gly-gly-ser-gly-gly-ser] (SEQ ID NO:3) that fuses the GF to the IgG domain is eliminated (direct fusions) or reduced to 2 to 4 amino acids can be constructed as described below. Similar methods can be used to create linkers shorter than 7 amino acids, to create linkers longer than 7 amino acids and to create linkers containing other amino acid sequences. The experiments described below use IgG1-Fc and EPO and G-CSF as examples, however, similar procedures can be used for other GF or IgG domains, and domains for other IgG subtypes and domains from IgM, IgA, IgD and IgE antibodies. The modified fusion proteins can be expressed, purified and their specific activities determined in *in vitro* bioassays as described in the Examples.--

The paragraph on page 24, lines 27-34, has been amended as follows:

--To construct a di-peptide [ser-gly] linker, one can PCR the IgG1-Fc sequence with a 5' oligonucleotide that adds the 5' extension CGCTCCGGA to the hinge coding sequence. The TCCGGA hexanucleotide is a cleavage site for the restriction endonuclease *Bsp* EI and encodes amino acids ser-gly. This PCR fragment can be digested with *Bsp* EI and *Sac* II and the ~ 240 bp fragment cloned into similarly cut pCDN3.1(+):EPO-IgG1-Fc and pCDNA3.1(+):G-CSF-IgG1-Fc. The unique *Bsp* EI site in each of these plasmids occurs at the first ser-gly in the linker [**ser-gly**-gly-ser-gly-gly-ser] (SEQ ID NO:3) so that the resulting recombinants will contain this 2 amino acid, ser-gly, linker. The sequence of the newly inserted ~ 250 bp *Bsp* EI-*Sac* II fragment can be verified.--

The paragraph spanning page 24, line 35 to page 25, line 3, has been amended as follows:

--A similar procedure can be used to construct the 4 amino acid [ser-gly-gly-ser] (SEQ ID NO:1) linker. One can PCR the IgG1-Fc sequence with a 5' oligonucleotide that

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adds the 5' extension CGCGGATCC to the hinge coding sequence. The GGATCC hexanucleotide is a cleavage site for the restriction endonuclease *Bam* HI and encodes amino acids gly-ser. This PCR fragment can be digested with *Bam* HI and *Sac* II and the ~ 240 bp fragment cloned into similarly cut pCDN3.1(+):EPO-IgG1-Fc and pCDNA3.1(+):G-CSF-IgG1-Fc. The unique *Bam* HI site in each of these plasmids occurs at the first gly-ser in the linker [ser-gly-gly-ser-gly-gly-ser] (SEQ ID NO:3) so the recombinants will contain the 4 amino acid (ser-gly-gly-ser) (SEQ ID NO:1) linker. The sequence of the inserted ~ 250 bp *Bam* HI – *Sac* II piece can be verified.--

The paragraph on page 40, lines 1-10, has been amended as follows:

--In order to construct and express gene fusions of IFN- $\alpha$ 2 with IgG coding sequences the IFN- $\alpha$ 2 gene was modified at the 5' and 3' ends using PCR based mutagenesis. pBBT160 plasmid DNA was used as template for PCR with forward primer BB108 (5' CGCAAGCTTGCCACCATGGCCTTGACCTTT GCTTTA-3'; SEQ ID NO:46) and reverse primer BB109 (5'- CGCGGATCCTCCGGATTCTTACTT CTAAACTTTC-3'; SEQ ID NO:47). Primer BB108 anneals to the 5' end of the coding sequence for the IFN- $\alpha$ 2 secretion signal and the reverse primer, BB109, anneals to the 3' end of the IFN- $\alpha$ 2 coding sequence. The resulting PCR product was digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that had been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. A clone with the correct DNA sequence (Henco et al., 1985) was designated pCDNA3.1(+):IFNAfus or pBBT190.--

The paragraph spanning page 51, line 32, to page 52, line 2, has been amended as follows:

--A GM-CSF-IgG1-Fc direct fusion can be created by PCR using plasmid pcDNA3.1::GM-CSF-IgG1-Fc as the DNA template. One PCR reaction can use oligos GMCSFDFA (5' GAGCCAGTCCAGGAGGAGCCCAAATCTTGTGACAAA-3'; SEQ ID NO:72) and BB82 (SEQ ID NO:14). The second PCR reaction can use oligos GMCSFDFB (5' ACAAGATTTGGGCTCCTCCTGGACTGGCTCCCAGCA -3; SEQ ID NO:73) and

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BB91 (SEQ ID NO:11). The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The approximate 1150 bp PCR product can be gel-purified, digested with *Hind* III and *Sac* II, the resulting ~ 675 bp *Hind* III/*Sac* II fragment gel-purified and cloned into similarly cut plasmid pcDNA3.1::GM-CSF-IgG1-Fc that had been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.--

The paragraph on page 52, lines 3-12, has been amended as follows:

--A GM-CSF-IgG4-Fc direct fusion can be created by PCR using plasmid pcDNA3.1::GM-CSF-IgG4-Fc as the DNA template. One PCR reaction can use oligos GMCSFDFC (5'-GAGCCAGTCCAGGAGGAGTCCAAATATGGTCCCCCA-3')(SEQ.ID.NO.[76] 74) and BB82. The second PCR reaction can use oligos GMCSFDFD (5'-ACCATATTTGGACTCCTCCTGGACTGGCTCCCAGCA-3')(SEQ.ID.NO.[77] 75) and BB91. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The approximate 1150 bp PCR product can be gel-purified, digested with *Hind* III and *Sac* II, the ~ 675 bp *Hind* III/*Sac* II fragment gel-purified and cloned into similarly cut plasmid pcDNA3.1::GM-CSF-IgG4-Fc that has been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.--

The paragraph spanning page 52, line 32, to page 53, line 11, has been amended as follows:

--9. A. Cloning Stem Cell Factor (SCF): SCF regulates development of hematopoietic progenitor cells. A cDNA encoding human SCF can be amplified by RT-PCR using RNA isolated from HepG2, 5637 or HT-1080 cell lines (Martin et al., 1990; the HepG2 and 5637 cell lines are available from the ATCC, Rockville, MD). PCR reactions can be carried out with forward primer SCF-F (5'-CGCAAGCTTGCCACCATGAAGAAGACACAAACT-3')(SEQ.ID.NO.[78] 76) and reverse primer SCF-R (5'-CGCGGATCCTCCGGAGTGTAGGCTGGAGTCTCCAGG

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–3')(SEQ.ID.NO.[79] 77). SCF DNA sequences in the primers are underlined. Primer SCF-F anneals to the 5' end of the coding sequence for the SCF secretion signal and the reverse primer, SCF-R, anneals to the 3' end of the SCF coding sequence, beginning at the junction of the extracellular and transmembrane domains. Other reverse PCR primers can be used to create truncated forms of the SCF extracellular domain, in particular a form that terminates following Ala-174 of the mature protein, by substituting appropriate nucleotides for the SCF DNA sequence listed in SCF-R. The resulting PCR product can be digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that has been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. Several clones can be sequenced to identify one with the correct DNA sequence. IgG1-Fc, IgG4-Fc, IgG1-C<sub>H</sub>, IgG4-C<sub>H</sub> and kappa light chain constant regions can be fused to the carboxy-terminus of the extracellular domain of SCF as described in Examples 1 and 5. The cell line TF-1 (Kitamura, 1989; available from the American Type Culture Collection, Rockville, MD) can be used to measure bioactivity of SCF-IgG fusion proteins.--

The paragraph on page 53, lines 12-22, has been amended as follows:

--Direct fusions of the extracellular domain of SCF to various IgG domains can be constructed using procedures similar to those described in Example 4 for constructing EPO-IgG and G-CSF-IgG direct fusions. A SCF-IgG1-Fc direct fusion can be created by PCR using plasmid pCDNA3.1::SCF-IgG1-Fc7AA as the DNA template. One PCR reaction can use oligos SCFDFF (5'-GACTCCAGCCTACACGAGCCCAAATCTTGTGACAAA-3') (SEQ.ID.NO.[80] 78) and BB82. The second PCR reaction used oligos SCFDFR (5'-ACAAGATTTGGGCTCGTGTAGGCTGGAGTCTCCAGG-3'; SEQ.ID.NO.[81] 79) and BB91. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The PCR product can be gel-purified, digested with *Hind* III and *Sac* II, and cloned into similarly cut pCDNA3.1::SCF-IgG1-Fc7AA that had been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.--

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The paragraph on page 53, lines 23-31, has been amended as follows:

--A SCF-IgG4-Fc direct fusion can be created by PCR using plasmid pcDNA3.1::SCF-IgG4-Fc7AA as the DNA template. One PCR reaction can use oligos SCFDFC (5'- GACTCCAGCCTACACGAGTCCAAATATGGTCCCCCA-3')(SEQ.ID.NO.[82] 80) and BB82. The second PCR reaction can use oligos SCDFD (5'- ACCATATTTGGACTCGTGTTAGGCTGGAGTCTCCAGG-3')(SEQ.ID.NO.[83] 81) and BB91. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The PCR product can be gel-purified, digested with *Hind* III and *Sac* II, and cloned into similarly cut pcDNA3.1::SCF-IgG4-Fc7AA that has been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.--

The paragraph spanning page 53, line 33, to page 54, line 13, has been amended as follows:

--**10. Flt-3L-IgG fusion Proteins:** Flt-3L (Lyman et al., 1993; Hannum et al., 1994) is a membrane bound cytokine that regulates development of hematopoietic stem cells. A cDNA encoding human Flt-3L can be amplified by PCR from single-stranded cDNA prepared from adult or fetal liver, kidney, heart, lung, or skeletal muscle, which is available from commercial sources such as CLONTECH and Stratagene, Inc.. PCR reactions can be carried out with forward primer fltF (5'- CGCAAGCTTGCCACCATGACAGTGCTGGCGCCAGCC-3')(SEQ.ID.NO.[84] 82) and reverse primer fltR (5'- CGCGGATCCTCCGGAAGGGGGCTGCGGGGCTGTCCG-3')(SEQ.ID.NO.[85] 83). Flt-3L DNA sequences in the primers are underlined. Primer fltF anneals to the 5' end of the coding sequence for the Flt-3L secretion signal and the reverse primer, fltR, anneals to the 3' end of the Flt3 coding sequence, beginning at the junction of the extracellular and transmembrane domains. Other reverse PCR primers can be used to create truncated forms of the Flt-3L extracellular domain by substituting appropriate nucleotides for the Flt-3L DNA sequence listed in primer fltR. The resulting PCR product

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can be digested with *Hind* III and *Bam* HI, gel purified and cloned into pCDNA3.1(+) vector that has been digested with *Hind* III and *Bam* HI, alkaline phosphatase treated, and gel purified. Several clones can be sequenced to identify one with the correct DNA sequence. IgG1-Fc, IgG4-Fc, IgG1-C<sub>H</sub>, IgG4-C<sub>H</sub> and kappa light chain constant regions can be fused to the carboxy-terminus of the extracellular domain of Flt-3L as described in Examples 1 and 5. Ba/F3 cells transfected with the human Flt-3 receptor (Lyman et al., 1993; Hannum et al., 1994) can be used to measure bioactivity of Flt-3L-IgG fusion proteins. Ba/F3 cells are available from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).--

The paragraph on page 54, lines 14-24, has been amended as follows:

--Direct fusions of the extracellular domain of Flt-3L to various IgG domains can be constructed using procedures similar to those described in Example 4 for constructing EPO-IgG and G-CSF-IgG direct fusions. A Flt-3L-IgG1-Fc direct fusion can be created by PCR using the pcDNA3.1::Flt-3L-IgG1 plasmid described above as the DNA template. One PCR reaction can use oligos FltDFA (5' - GCGCCGAGCCCCCTGAGCCCAAATCTTGTGACAAA-3' (SEQ.ID.NO.[86] 84) and BB82. The second PCR reaction can use oligos Flt3DFB (5' - ACAAGATTTGGGCTCAGGGGGCTGCGGGGCTGTCTGG-3' (SEQ.ID.NO.[87] 85) and BB91. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The PCR product can be gel-purified, digested with *Hind* III and *Sac* II, and cloned into similarly cut pcDNA3.1::SCF-IgG1-Fc7AA that had been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.--

The paragraph on page 54, lines 25-32, has been amended as follows:

--A Flt-3L-IgG4-Fc direct fusion can be created by PCR using plasmid pcDNA3.1::Flt3-IgG4-Fc7AA as the DNA template. One PCR reaction can use oligos FltDFC (5' - GCGCCGAGCCCCCTGAGTCCAAATATGGTCCCCCA-

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3')(SEQ.ID.NO.[88] 86) and BB82. The second PCR reaction can use oligos FltDFD (5'-ACCATATTTGGACTCAGGGGGCTGCGGGGCTGTCGG-3')(SEQ.ID.NO.[89] 87) and BB91. The products from these PCR reactions can be gel-purified, mixed and subjected to a third PCR reaction using oligos BB82 and BB91. The PCR product can be gel-purified, digested with *Hind* III and *Sac* II, and cloned into similarly cut pcDNA3.1::SCF-IgG4-Fc7AA that has been treated with calf intestinal phosphatase. A clone with the correct insert can be identified by DNA sequencing.--

In the Claims:

Claims 4-8, 12, 14, 17, 20-26, 28, 32, 34, 37, 38 and 42 have been amended as shown below.

Claims 1-3, 9, 10, 15, 16, 18, 19, 27, 29-31, 33, 35, 36, and 39-41 remain unchanged.

Claims 43-61 have been added.

4. (Once Amended) The fusion protein of claim 2, wherein the peptide linker is Ser(GlyGlySer)<sub>n</sub> (SEQ ID NO:1), wherein n is 1 to 7.

5. (Once Amended) The fusion protein of claim 2, wherein the peptide linker is Ser([GlyGlySer]) (SEQ ID NO:1) or Ser(GlyGlySer)<sub>2</sub> (SEQ ID NO:3).

6. (Once Amended) The fusion protein of [any one of claims 1-5] Claim 1, wherein the Ig domain is selected from the group consisting of IgG-Fc, IgG-C<sub>H</sub> and IgG-C<sub>L</sub>.

7. (Once Amended) The fusion protein of [any one of claims 1-5] Claim 1, wherein the soluble protein is a member of the growth hormone (GH) supergene family.

8. (Once Amended) The fusion protein of [any one of claims 1-5] Claim 1, wherein the soluble protein is granulocyte-colony stimulating factor (G-CSF).

12. (Once Amended) The fusion protein of [any one of claims 1-5] Claim 1, wherein the soluble protein is growth hormone (GH).

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14. (Once Amended) The fusion protein of [any one of claims 1-5] Claim 1, wherein the soluble protein is selected from the group consisting of granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-11 (IL-11), thrombopoietin (TPO), stem cell factor (SCF) and flt3 ligand.

17. (Once Amended) The homomultimeric fusion protein of [any one of claims 15-16] Claim 15, wherein the member of the GH supergene family is granulocyte-colony stimulating factor (G-CSF).

20. (Once Amended) The homomultimeric fusion protein of [any one of Claims] Claim 19 [or 40], wherein the multimeric fusion protein is a dimeric EPO fusion protein.

21. (Once Amended) The homomultimeric fusion protein of [any one of claims 15-16] Claim 15, wherein the member of the GH supergene family is selected from the group consisting of: growth hormone, alpha interferon, beta interferon, gamma interferon, GM-CSF, IL-11, TPO, SCF, and Flt3 ligand.

22. (Once Amended) The fusion protein of [any one of claims 15,] Claim 16[, 40 or 41], wherein the peptide linker is SerGly.

23. (Once Amended) The fusion protein of [any one of claims 15,] Claim 16[, 40 or 41], wherein the peptide linker is Ser(GlyGlySer)<sub>n</sub> (SEQ ID NO:1), wherein n is 1 to 7.

24. (Once Amended) [A purified] The fusion protein [according to any one of Claims] of Claim 1 [ or 2], wherein said [the purified] fusion protein is dimeric and is essentially free of monomeric fusion protein.

25. (Once Amended) The [purified] fusion protein of claim 24, wherein the soluble protein is selected from the group consisting of G-CSF, EPO and interleukin-11.

26. (Once Amended) A method of producing a fusion protein [according to any one of Claims 1,] of Claim 2[, 15, 16, 20 or 41], comprising:

- a. transfecting or transforming a host cell with at least one nucleic acid encoding [an immunoglobulin domain and a soluble protein selected from the group

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consisting of a growth factor, a cytokine, and an active variant thereof] the fusion protein of Claim 2;

- b. culturing the host cell; and
- c. harvesting the fusion protein expressed by the host cell.

28. (Once Amended) A nucleic acid encoding the fusion protein of [any one of Claims] Claim 1[, 2, 15, 16, 40 or 41].

32. (Once Amended) A method of purifying the fusion protein [according to any one of Claims] of Claim 1[, 2, 15, 16, 40 or 41], comprising:

- a. obtaining a composition comprising the fusion protein; and
- b. isolating the fusion protein from contaminants by column chromatography.

34. (Once Amended) A method of treating a condition treatable with a member of the Growth Hormone (GH) supergene family, comprising administering an effective amount of the fusion protein of [any one of Claims] Claim 1[, 2, 15, 16, 40 or 41] to a patient in need thereof.

37. (Once Amended) A pharmaceutical composition comprising the fusion protein of [any one of Claims] Claim 1[, 2, 15, 16, 40 or 41] in a pharmaceutically acceptable carrier.

38. (Once Amended) The fusion protein of [one of Claims] Claim 1 [or 2], wherein the soluble protein is erythropoietin (EPO).

42. (Once Amended) The method of Claim 26, further comprising purifying [the] dimeric fusion protein from monomeric fusion protein.